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EFFECTS OF HYDROGEN ION CONCENTRATION ON THE MORPHOLOGY OF HEMOCYTES OF THE MOLE-CRAB *EMERITA ASIATICA*

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In previous studies (Ravindranath, 1973, 1974a, b) attempts have been made to extend Jones's classification of hemocytes of insects to the hemocytes of non-insect arthropods, for this scheme is based on morphological criteria. Recently some investigators expressed the view that morphologically different types of hemocytes may represent developmental and functional phases of a single basic cell type (Scharrer, 1972; Price and Ratcliffe, 1974). Gupta and Sutherland (1966) suggested that physiological changes in the hemolymph alter the morphology of hemocytes to a considerable extent, but very little experimental work has been undertaken to verify this suggestion. Such an attempt to analyze hemocyte morphology under different experimental conditions is undoubtedly required before any truly objective definition of hemocyte morphology can be given.

Accidental observations indicate that alteration of the pH of the surrounding medium of the hemocytes may bring about a change in the morphology and behavior of the hemocytes (Grégoire, 1953; McLaughlin and Allen, 1965; Noble, 1970). The present study aims at studying the effects of pH, if any, on the morphology and behavior of hemocytes of *Emerita asiatica*.

Materials and Methods

Specimens of the mole-crab, *Emerita asiatica*, collected from a sandy shore were used within six hours, and observations were made on sexually mature females with carapace length ranging from 30 to 40 mm.

All animals were bled by amputation of the first thoracic appendage, thus minimizing the variations in the blood picture which can result from the use of different bleeding techniques (Jones, 1962). Care was taken to avoid mixing of sand particles or sea water from the appendage with the blood sample.

For studying the effect of pH on the morphology of hemocytes the procedure employed by Grégoire (1953) and McLaughlin and Allen (1965) was followed. A drop of hemolymph issuing from the severed leg was allowed to fall in approximately twice its volume of buffer solution placed into the middle of a glass slide. A thin coverslip, wet in its middle part with the buffer at the pH tested, was gently placed over the hemolymph-buffer mixture. Spontaneous spreading gave a film of suitable thinness for observation. With this procedure, Grégoire (1953) noted that the labile hemocytes are protected from contact with glass surfaces before being thoroughly mixed with the test solution. Control preparations were made with bidistilled water, in order to appreciate the part played by the dilution of the hemolymph and by the pH in the modification of the hemocyte pictures. Unavoidably, the above method of analysis introduced three factors that could

possibly influence the results: (1) exposure of the hemolymph to air, (2) the pressure of the coverslip, and (3) osmotic and ionic conditions. The exposure to air was minimized by bringing the cut end of the appendages quickly in contact with the drop of buffer already on the slide. The effect of coverslip pressure on the hemocytes appears to be negligible, for, the changes in the morphology of hemocytes were observed to commence only after about two minutes in all preparations. Besides, uniform trends in the results were obtained in all the six specimens examined with reference to each pH. The buffer systems employed in the present study include Sörensen's M/15 Phosphate buffer, 0.2 M Acetate buffer and 0.02 M Tris-HCl buffer. The major observations were made using Tris-HCl buffer system. One of the advantages of this buffer system is that the buffer solutions of varying pH can be prepared without altering the ionic strength appreciably (Bates, 1961).

The experimental analysis with reference to each buffer series was repeated twice. Each time, three animals were used for every pH of the given buffer. After analyzing the hemolymph with different levels of pH for any particular buffer this way, the analysis was repeated again for the same buffer series. One advantage in experimenting in this way lies in the capacity to carry out detailed observations and thereby detect features which might be overlooked in attempted analysis of larger samples (Arnold, 1969). This method of analysis also facilitates detection of any vague responses. In the present study, the response in question is a shift in the composition of the hemocyte population, which is detectable only by means of differential hemocyte counts.

Differential hemocyte counts were made by classifying a minimum of 200 cells per animal, and the counts were made continuously for ten minutes. The time of addition of hemolymph to buffer solution is taken as zero minute. For studying the effect of pH on aggregation of hemocytes the procedure of Noble (1970) was followed. The hemolymph-buffer mixture was allowed to stand for ten minutes. The number of cell aggregates and the number of cells that were not associated with a cell aggregate, that is, the free cells, were determined. The ratio was expressed as a percentage of total cells.

For interpretation of results, the pH of the hemolymph and pH of the hemolymph-buffer mixture were determined. The hemolymph oozing from the cut end of the appendage was drawn directly into the glass electrode of the pH meter. By this procedure, pH rise due to loss of carbon dioxide is reduced (Stewart, Dingle and Odense, 1966; Bailey, 1971). The influence of dilution of hemolymph with buffers on coagulation of the hemolymph was found out by determining the clotting time, following the procedure described by Peters and Long (1973). All the experiments were carried out between 28° C to 30° C.

Results

Initially, under phase-contrast optics, two categories of hemocytes were distinguished in a fresh drop of hemolymph of *Emerita asiatica*. One type that constitutes about 65% to 75% of the total cells was characterized by the numerous boat-shaped refractile granules. This cell type varied in its length from 18 to 25 μ. The nucleus was spherical and centrally situated. Its presence was sometimes masked by the mass of refractile granules. The other type constituted about

25% to 35% of the total cells, was non-refractile, round, and balloon-like, with a small, round, cart-wheel-like and usually eccentric nucleus. This type fits the description of explosive corpuscles of Hardy (1892) and cystocytes of Jones (1962). Besides these two categories, other types comparable to prohemocytes, plasmatocytes, spherule cells and adipohemocytes of insects (Jones, 1962) were rarely seen (Ravindranath, 1975). All these rare types did not occur in the hemolymph of a single animal.

Effects of pH on the hemolymph

The pH of the hemolymph of intermolt *Emerita asiatica* is 7.95 ± 0.12 at 30° C. This value is in general agreement with pH values of the hemolymph of several decapods (Mangum and Shick, 1973). The pH of the hemolymph-buffer mixture (1 : 2 v/v) varied very slightly with different buffer systems. In Tris-hemolymph mixture, the pH was essentially the same as that of the respective pH value of the buffer system.

In fresh hemolymph, gelification of plasma occurred within 3 minutes at 30° C. The hemolymph-buffer mixture showed variable results. There was no plasma gelification in the acidic range of the pH. Above pH 7.0 gelification is slow and incomplete. The cytoplasmic veils, characteristic of the second pattern of coagulation, described by Grégoire (1970), built up by some disintegrating cystocytes, were infrequently detected by their stretched folds, in the alkaline range of pH. In sodium acetate buffer systems (pH 2.6 to 5.0), the hemolymph precipitated intensely. The precipitation of hemolymph was low or absent in all the pH ranges of phosphate and Tris-HCl buffer systems, respectively.

Effects of pH on aggregation of hemocytes

The results (Fig. 1) show that the percentage of cell aggregates increases with increase in pH of Tris-HCl buffer system.

Effects of pH on the hemocytes

Comparison of hemocytes in preparations diluted with the different pH ranges of buffers, with those of fresh, undiluted or distilled water diluted preparations, gave clear indications of alteration of granular hemocytes in relation to variations in pH. The cystocytes swell and disintegrate in the solution within 3 min. In acetate buffers at pH 3.6, 4.0, 4.4, 4.8, 5.2, and 5.6, and in the phosphate buffers at a range between 6.0 and 6.8, the granular hemocytes underwent a very rapid alteration to spindle-shaped and pseudopodial forms. The refractile boat-shaped granules became fine. The homogeneous appearance of the nucleus changed into heterogeneous.

In phosphate buffer at pH 7.2, 7.6, and 7.8 as well as in all ranges of Tris-HCl buffer, the hemocytes underwent similar changes, but the speed with which the alterations occurred differed with different hydrogen ion concentrations and buffer systems.

Stages of transformation

The alterations observed in the granular hemocytes are classified as follows: *Stage I* (Figs. 2 and 3)—this stage is represented by the unaltered amoeboid

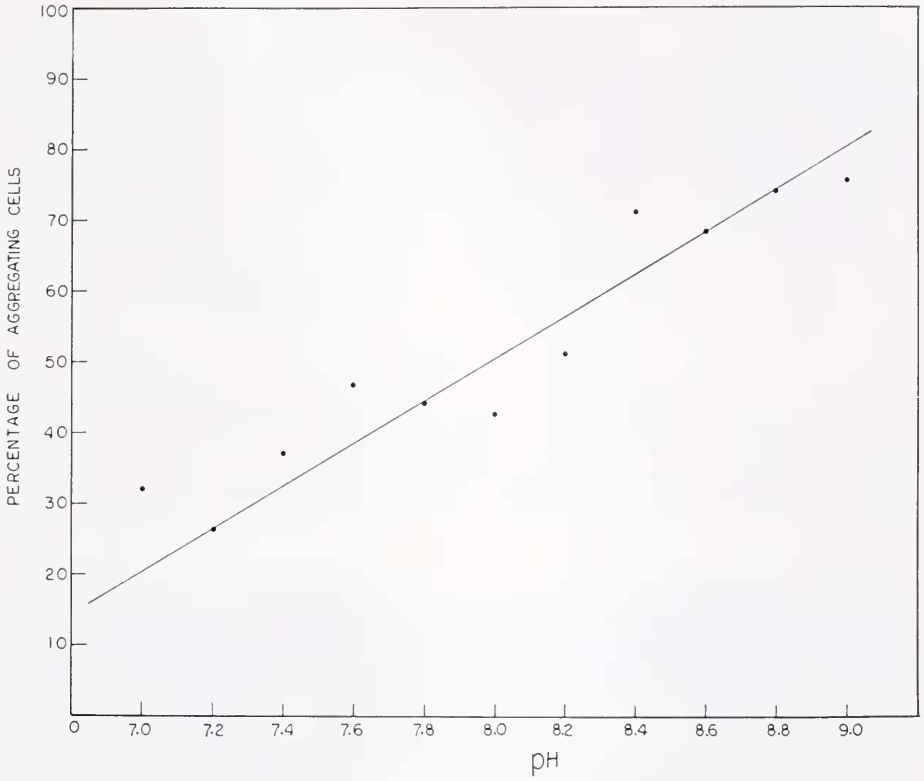

FIGURE 1. The percentage of aggregating hemocytes in different hydrogen-ion concentrations of 0.02 M Tris-HCl buffer system. The ratio of the number of hemocyte aggregates and the number of free cells are expressed as percentage of total cells.

granular hemocytes. *Stage II* (Figs. 2 and 4)—in this stage the boat-shaped refractile granules became fine, and some lose their refractility. The nucleus was visible and appeared to be homogeneous. The cells continued to be amoeboid. *Stage III* (Figs. 2 and 5)—in this stage the granules disappeared completely. The nucleus was clearly visible, and the cells exhibited amoeboid movements in buffer-hemolymph mixtures. Cells of this stage were identical to plasmatocytes described by previous workers (see Jones, 1962). *Stage IV* (Figs. 2 and 6)—in this stage the cell became round and appeared balloon-like, due to vacuolization of the cytoplasm. The nucleus shrunk in its size and became pycnotic, with a reticular chromatin structure, indicative of chromatin disaggregation. The cells were amoeboid. All these features of the cell strongly recall those of the cystocytes. In some preparations, between the buffer range of 8.6 to 9.0, it was noted that the cart-wheel-like nucleus again turned homogeneous and showed distinct signs of swelling of the nucleus. In some cases, cytoplasm was observed to shrink and the cell gave the appearance of a prohemocyte. In the present study such cells were also included in Stage IV. Overlapping of the stages was observed near neutral pH but it does not occur frequently.

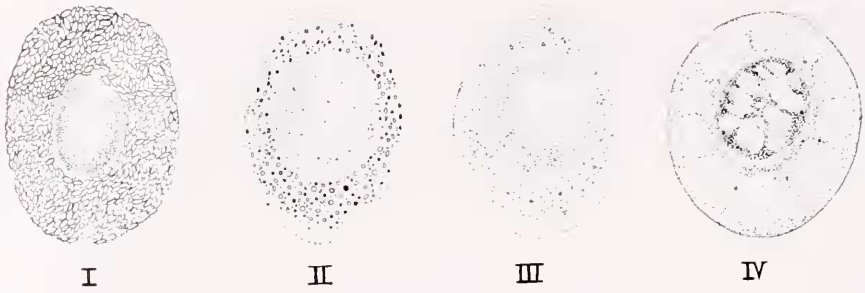

FIGURE 2. The sequential changes observed in a granular hemocyte in the pH range between 7.0 and 9.0 of Tris-HCl buffer. The changes are arbitrarily classified into four stages: I) normal granular hemocyte in unaltered condition in the buffer; II) the second stage of the hemocyte transformation, note the dissolution of the elliptical granules; III) the third stage recalls a plasmatocyte, note the degranulation of cytoplasm; and IV) the final stage recalls a cystocyte, note the vacuolization of the cytoplasm, stiffening of the cell surface and nuclear pycnosis.

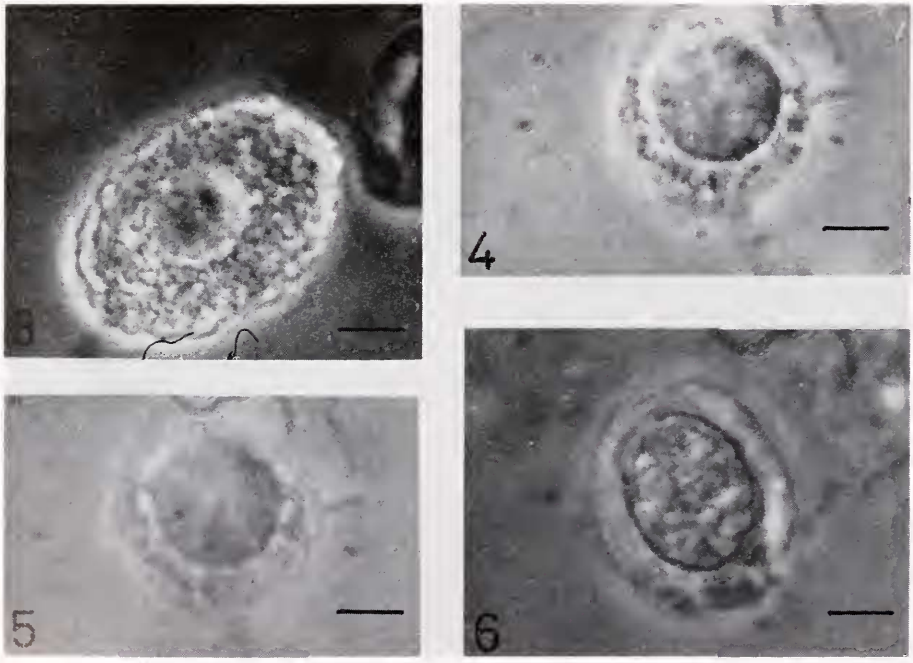

FIGURE 3. Normal granular hemocyte in 0.02 M Tris-HCl buffer at pH 8.2. Note the refractile and elliptical granules and homogeneous nucleus.

FIGURE 4. The second stage of granular hemocyte transformation in Tris-HCl buffer at pH 8.2. Note the dissolution and the loss of refractility of the elliptical granules.

FIGURE 5. The third stage. Note the degranulation of the cytoplasm.

FIGURE 6. The last stage. Note the vacuolization of the cytoplasm, stiffening of the cell surface and the nuclear pycnosis.

Differential counts of the stages of hemocytes

Initially, the differential counts of the above referring to stages of the granular hemocytes were made with reference to phosphate buffer (pH 7.0, 7.6, 7.8, and 8.0). Precipitation of hemolymph in this buffer and the resultant clumping of hemocytes with the precipitate did not permit a detailed analysis. Therefore, the differential counts were made in detail with Tris-HCl buffer system in which precipitation was absent. Moreover, as the ionic strength of Tris-HCl buffer solutions of varying pH is not altered appreciably, the ionic effects of the buffer on the hemocytes may not be significant. The results (Fig. 7) show that the alterations in the morphology of granular hemocytes varied significantly with different hydrogen ion concentrations and times. As the pH of the solution increased from 7.0 to 9.0, the transformation of granular hemocytes slowed down. Correlated with the increase in transformation at pH 7.6, a marked increase in the cystocyte-like cells was observed (Fig. 7). The overall pattern of fluctuations of cystocyte-like cells (Stage IV cells) showed an inverse trend to those of granular hemocytes and cells of Stages II and III. When granular hemocytes and cells of stages II and III showed a maximum retardation in their alteration at pH 8.2, the percentage of Stage IV cells decreased significantly. At all levels of pH, cells of all stages except Stage IV, showed a decrease in their number after ten minutes (Fig. 7).

Discussion

The most striking feature in the composition of the hemocytes of *Emerita asiatica* is the predominance of one cell type, namely the granular hemocyte. A characteristic feature of this hemocyte is the nature of granular inclusions, which are refractile and boat-shaped. This type of hemocyte has been reported in several arthropods (see Ravindranath, 1975). But such a cell type does not fall in any of the categories of hemocytes classified by Jones (1962). His description and the figures of the granular hemocytes (Jones, 1962, 1965) and the granular hemocytes reported in a millipede, *Thyropygus poseidon* (Ravindranath, 1973), correspond to Stage II cells of the present study.

Arnold (1959, 1972), on the other hand, has observed the refractile boat-shaped granules both in plasmatocytes (Fig. 12, 1959; Fig. 104, 1972) and in granular hemocytes (Figs. 105 and 123, 1972) of *Blaberus discoidalis* and *B. giganteus*. Such a striking similarity in the granulation of the cytoplasm of these two different categories of cells emphasizes the need for an objective, unambiguous distinction of plasmatocytes and granular hemocytes (see also discussion in Ravindranath, 1974a). It appears therefore that the classification of hemocytes of arthropods is still in an incomplete stage.

The observations made in the present study with reference to the influence of pH indicate that the pH of the surrounding medium influences both the behavior and the morphology of hemocytes. Comparison of the results with earlier observations is hampered by the fact that previous analyses are predominantly qualitative. However, the observations made here not only confirm the findings of others (Cameron, 1952; Loeb and Blanchard, 1922; Grégoire, 1953; McLaughlin and Allen, 1965; Noble, 1970), but also favor the suggestion of McLaughlin and

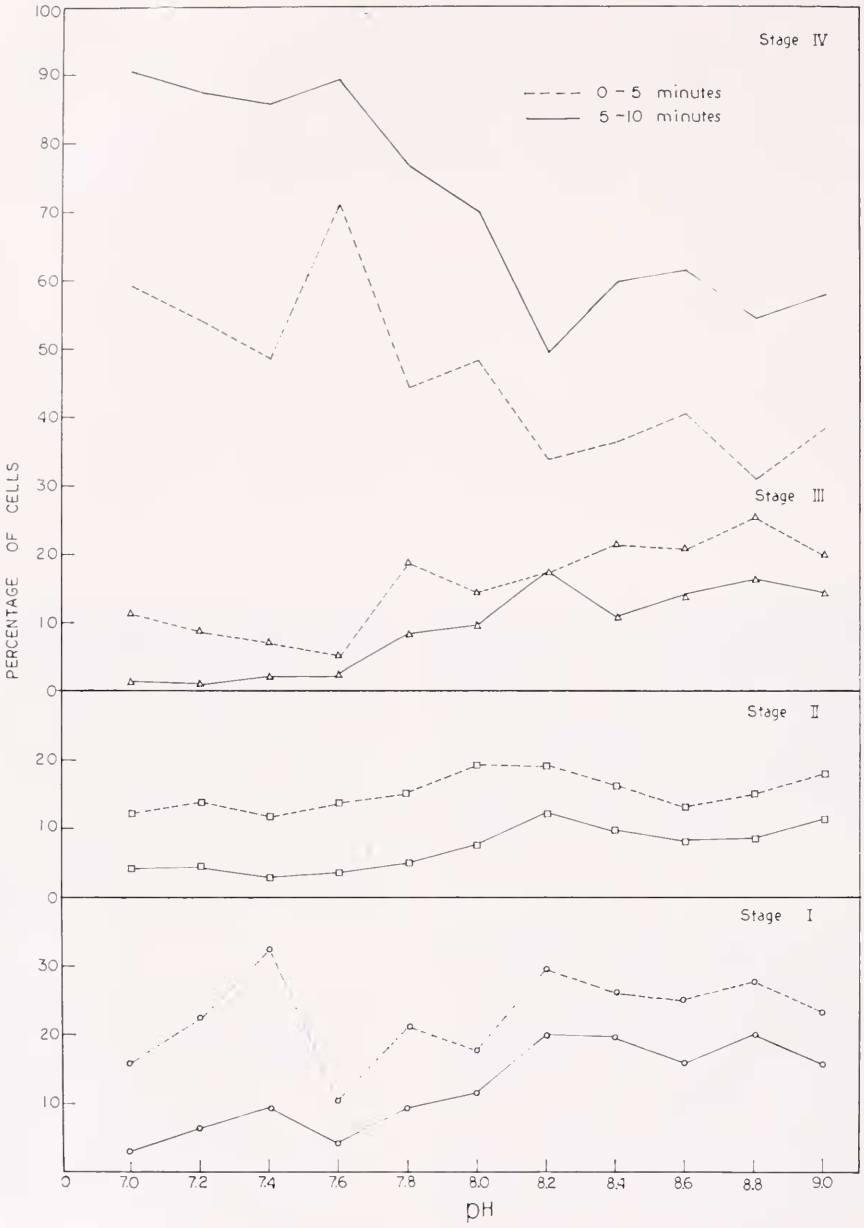

FIGURE 7. The response of granular hemocytes to variation in hydrogen-ion concentrations of Tris-HCl buffer. The response is detected by means of differential counts of the stages, which is expressed as percentage of total cells.

Allen (1965) that attention to the pH of diluents is important in a study of hemocyte morphology.

Some important features could be made with reference to the pH-induced morphological variations of granular hemocytes, though the experimental conditions may not reflect a true physiological state of the animal. First of all, the pH-induced mutability of granular hemocytes is not at random but sequential; secondly, the stages of alterations of hemocytes are facsimiles of the major arthropod hemocyte types, namely, granular hemocytes (described by Jones, 1962, 1965), the plasmatocytes and cystocytes. The above features suggest that the specific physiological conditions of the animals which bring about a change in hemolymph pH, such as autotomy and molting (Bliss, 1960) may likewise trigger the transformation of one form of hemocyte into another. Although such a suggestion is in general agreement with the views of Gupta and Sutherland (1966), the fact that there are inherent dangers in extending the observations based on *in vitro* experiments to *in vivo* behavior, necessitates further experimental data to confirm the above suggestion.

A feature of significant interest is the formation of cystocyte-like cells in the final stages of transformation of granular hemocytes. This transformation is brought about by degranulation and vacuolization of the cytoplasm and nuclear pycnosis, which may be a result of cell injury or toxicity, or autolysis (Dernby, 1918) caused due to alteration in the pH of the surrounding medium. The effect is minimized at pH 8.2. The above mentioned effects of cell injury or autolysis are also known to be caused as a result of aging of cells (Myers and DeWolfe-Glade, 1964). It is reasonable to infer that cystocytes which also occur in the normal hemolymph of *Hippa* (*Emerita asiatica*) and of other arthropods may be the "end-cell stage" of aging granular hemocytes (see also discussion in Ravindranath, 1975).

The effects of pH on the morphology of cells in vertebrates is well known. The works of Meyer and Sievers (1936), Teorell (1951) and Bittar (1964) indicate that the variations in pH may affect the stability and permeability of hemocyte membranes by altering the strength of the charges of the solution of protein, which exist in and out of the cells. Probably the increase in aggregation of hemocytes with increase in pH could be due to similar alterations in the properties of the cell surface.

An important work that is relevant to the observations made in the present study is that of Dernby (1918), who first applied Sörensen's concept of pH and made an extensive study of autolysis of several tissues as a function of pH, recognizing the existence of an acid optimum and also a second one in the alkaline range. It is evident from the above work, that the observed alterations in the morphology of hemocytes may be indicative of autolysis. The overall pattern of fluctuations of cystocyte-like cells (Fig. 7) is noteworthy in this connection for it indicates increased rate of transformation (autolysis?) of granular hemocytes at two points, one in the acid and the other in the alkaline region.

However it may be, the above results necessitate the importance of determining the pH of the hemolymph wherever different hemocyte counts are made, for the variations observed during different physiological conditions of an arthropod (see Ravindranath, 1974c) could be due to the variations in the pH of the hemolymph that occur during such periods rather than due to the different physiological requirements *per se*.

In conclusion, it is realized that classification of hemocytes into arbitrary types is necessary for purposes of quantitative morphological or physiological investigations, even if the various hemocytes should be found to represent only phases of development of single types (Jones, 1962). Moreover, the different developmental phases of a single cell type could be capable of distinct functions just as truly different cell types are (Price and Ratcliffe, 1974). While classifying the cells it should be noted that a classification based on prepared hemolymph films alone, which has been the common practice, or from any single technique, seems unwise and would definitely mislead in the interpretation of the hemocyte complex of arthropods (see also discussion in Arnold, 1959).

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Summary

Variations in the pH of the surrounding medium of hemocytes *in vitro* influence the morphology of granular hemocytes, the most predominant cell type in the hemocyte population of *Emerita asiatica*. Qualitative studies reveal that in the acidic range these cells become spindle-shaped and pseudopodial. Between pH 7.0 and 9.0 of the Tris-HCl buffer system, the hemocytes show a sequential change. The morphological features of the stages are facsimiles of the major hemocyte types occurring in the hemolymph of *Emerita asiatica*. The granular hemocytes become plasmatocyte- and cystocyte-like cells.

Differential counts of hemocytes and pH-induced variations of granular hemocytes reveal that the rate of change decreases with decreasing pH of the surrounding medium. The aggregation of hemocytes increases with increase in pH.

The pH-dependent variations of hemocytes indicate that for a truly objective definition of hemocyte morphology and for a strict classification into distinctly separate cell types, experimental analyses of hemocyte morphology are required.

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